Nature Conservation 56: 19–36 (2024)
DOI: 10.3897/natureconservation.56.111745

Research Article

Phylogeography and genetic population structure of the endangered bitterling *Acheilognathus tabira tabira* Jordan & Thompson, 1914 (Cyprinidae) in western Honshu, Japan, inferred from mitochondrial DNA sequences

Gen Ito[1,2], Naoto Koyama[3], Ryota Noguchi[2], Ryoichi Tabata[4], Seigo Kawase[4], Jyun-ichi Kitamura[2,5], Yasunori Koya[6]

1 *Center for Biodiversity Science, Ryukoku University, 1-5 Yokotani, Seta Oe-cho, Otsu, Shiga 520-2194, Japan*
2 *Japan Watershed Conservation Network, 1281 Chuma-cho, Matsusaka, Mie 519-2143, Japan*
3 *TAKAYASU Study group of Japanese rose bitterling, 4-28 Koorigawa, Yao, Osaka 581-0872, Japan*
4 *Lake Biwa Museum, 1091 Oroshimo, Kusatsu, Shiga 525-0001, Japan*
5 *Mie Prefectural Museum, 3060 Isshinden-kouzubeta, Tsu, Mie 514-0061, Japan*
6 *Faculty of Education, Gifu University, 1-1 Yanagido, Gifu 501-1193, Japan*
Corresponding author: Gen Ito (sakurahayabusa6647@gmail.com)

Abstract

We examined the genetic population structure of the endangered freshwater cyprinid *Acheilognathus tabira tabira* in the Japanese archipelago, which has only been analyzed in limited sampling in previous studies, based on cytochrome *b* region of the mitochondrial gene. We confirmed the existence of the same three lineages determined in the previous study, the natural distribution area of Lineage I and II+III were considered to be the Seto Inland Sea and Ise Bay regions, respectively. Furthermore, the Seto Inland Sea region population was divided into five groups inhabiting neighboring water systems using the spatial analysis of molecular variance (SAMOVA). We estimated that populations in the Seto Inland Sea region migrated through a single paleowater system during the last glacial period and were then separated and genetically differentiated due to marine transgression. The Yoshino River system population was estimated to be a non-native population because it belonged to the same group as the Lake Biwa-Yodo River system, which is the only separate water system across the Seto Inland Sea. This study provides new evidence of genetic differentiation in *A. t. tabira* populations within the Seto Inland Sea region, where genetic differentiation has not been detected in previous studies, corresponding to five different groups by significantly increasing the number of individuals and sites compared with previous studies. Therefore, we propose these five groups as conservation units in the Seto Inland Sea region.

Key words: Artificial introduction, biogeography, conservation, Cytochrome *b*, SAMOVA

OPEN ACCESS

Academic editor: Valter Azevedo-Santos
Received: 28 August 2023
Accepted: 4 July 2024
Published: 8 August 2024

ZooBank: https://zoobank.org/94926935-4515-4A64-80DD-990C35832BB1

Citation: Ito G, Koyama N, Noguchi R, Tabata R, Kawase S, Kitamura J-i, Koya Y (2024) Phylogeography and genetic population structure of the endangered bitterling *Acheilognathus tabira tabira* Jordan & Thompson, 1914 (Cyprinidae) in western Honshu, Japan, inferred from mitochondrial DNA sequences. Nature Conservation 56: 19–36. https://doi.org/10.3897/natureconservation.56.111745

Copyright: © Gen Ito et al.
This is an open access article distributed under terms of the Creative Commons Attribution License (Attribution 4.0 International – CC BY 4.0).

Introduction

The distribution of many freshwater fishes of the Japanese archipelago has been strongly influenced by geomorphic changes such as uplift of mountains (e.g., Watanabe et al. 2017). In particular, western Japan, including the Ise Bay and Seto Inland Sea regions, was significantly affected by mountainous uplift and transgression during the Pleistocene (Ota et al. 2004; Machida et al. 2006). It is known that these geomorphic changes have caused populations of freshwater fish to become fragmented and genetically differentiated within localized areas (Watanabe et al. 2017). Previous phylogeographic and genetic population structure studies of freshwater fishes in these regions suggest that geological events, such as uplift of mountains and marine transgression that influence the distribution of freshwater fishes may differ among species (Kitagawa et al. 2001; Watanabe et al. 2010, 2014; Tominaga et al. 2016, 2020; Nakagawa et al. 2016; Ito et al. 2019; Ito and Koya 2022). Identifying the phylogeographic patterns and genetic population structure of each species inhabiting the same region is an effective approach to identify the factors shaping the genetic diversity of freshwater fish (Avise 2000).

The tabira bitterling, *Acheilognathus tabira* Jordan & Thompson, 1914 (Cyprinidae: Acheilognathinae), is a freshwater fish endemic to Honshu, Japan. It has been classified into five subspecies, mainly due to their different nupital color patterns (Arai et al. 2007). Each subspecies has an allopatric distribution, differs in egg shape, and is clearly distinguishable by mitochondrial DNA (mtDNA) and nuclear DNA (Arai et al. 2007; Kitamura et al. 2012). Therefore, each subspecies is thought to have followed its own evolutionary path. The white tabira bitterling, *Acheilognathus tabira tabira* Jordan & Thompson, 1914, is a subspecies of *A. tabira*. The natural distribution range of *A. t. tabira* is the Ise Bay waters and the Seto Inland Sea regions on the Pacific side, with some rivers flowing into the Sea of Japan in western Japan (Kitamura and Uchiyama 2020). Previous phylogeographical studies of *A. t. tabira* have identified three lineages (Kitamura et al. 2012; Umemura et al. 2012), of which two are thought to be naturally distributed in the Ise Bay region (Nobi Plain groups I and II), and one lineage is thought to occur in the Seto Inland Sea region (Kinki-Sanyo group). However, the populations of the Seto Inland Sea region analyzed in previous studies were restricted to only two river systems; therefore, knowledge of the ranges of natural distribution in each lineage of *A. t. tabira* is incomplete.

In addition, *A. t. tabira* is listed as Endangered on the Red List of Japan because its population has been decreasing owing to improvements in rivers and agricultural canals (Ministry of the Environment of Japan 2020). However, the Kinki-Sanyo group has been artificially introduced into several regions as a result of incidental introductions associated with fishery releases of *Plecoglossus altivelis altivelis* (Temminck & Schlegel, 1846) and private releases, which raises concerns about the impact of genetic introgression on the native population of *A. t. tabira* (Umemura et al. 2012; Kumagai and Hagiwara 2013; Kitamura and Uchiyama 2020; Ito et al. 2021). In the Ise Bay region, the non-native lineage known as the Kinki-Sanyo group has been artificially introduced into the Nagara, Kiso, and Kushida Rivers, which are the natural distribution areas of the Nobi Plain Groups I and II (Kitamura et al. 2012; Umemura et al. 2012; Kitamura and Uchiyama 2020). Therefore, the native population of *A. t. tabira* in the Ise Bay region may have

become extinct due to genetic introgression (Kitamura and Uchiyama 2020). In the Yoshino River system in northeastern Shikoku, located in the Seto Inland Sea region, the past freshwater fish fauna is unknown; therefore, it has been difficult to determine whether the population of *A. t. tabira* is naturally distributed or originated from artificial introduction (Kitamura and Uchiyama 2020). Phylogeographic patterns and genetic population structures can help identify whether the population is naturally distributed or originates from non-native populations (Miyake et al. 2011; Umemura et al. 2012; Matsuba et al. 2014; Uemura et al. 2018; Tominaga et al. 2020; Ito et al. 2021, 2022). Therefore, understanding the phylogeographic patterns and genetic population structure across the natural distribution of *A. t. tabira* is essential for promoting appropriate conservation activities.

In the present study, we attempted to elucidate the factors responsible for distribution patterns of *A. t. tabira* by estimating its phylogeographic and genetic population structures covering its whole distribution range using the cytochrome *b* (cytb) region of the mtDNA. In addition, we discuss the artificial introduction and conservation units of *A. t. tabira* based on the results obtained.

Materials and methods

Sample collection

In total, 140 individuals were collected from 12 localities in 10 river systems in the Seto Inland Sea and Ise Bay regions from 2015 to 2020 (Fig. 1, Suppl. material 1), covering the whole geographic range of *A. t. tabira*. The unsampled sites in this study are the Nagara, Kiso, and Kushida river systems investigated by previous studies (Kitamura et al. 2012; Umemura et al. 2012), and rivers that flow into the Sea of Japan, which are probably extinct. For the populations in the Nagara, Kiso, and Kushida River, we cited base sequence data from previous studies (Kitamura et al. 2012; Umemura et al. 2012, Table 1). Additionally, a captive population from the Gifu World Freshwater Aquarium was included in this study. This captive population is believed to have originated from a population collected and cultured by a citizen from the Nagara River system in the Ise Bay area, which was donated to the Gifu World Freshwater Aquarium in 2004 (Koki Ikeya, personal communication). The mtDNA lineage of this population is described as native to the Ise Bay region by Mukai (2019). However, it is not indicated whether it belongs to the Nobi Plain group I or II. Individual bitterling were collected using hand nets and fishing methods. Each specimen was subjected to caudal fin clipping, and the remaining specimens were fixed in 10% formalin and preserved in 70% ethanol. For habitat conservation, the number of individuals collected was limited to 20 or fewer. The fin clips were preserved in 99% ethanol and stored at −20 °C. The specimens were registered with the Gifu and Mie Prefectural Museum along with collection site information (GPM-Z-22109, MIE-Fi3500, 3506–3512, 3519–3534, 3536–3540, 3543–3579, 3581–3604, 3606–3624, 3626, 3630–3633, 3718, 4272).

mtDNA analysis

Total genomic DNA was extracted from a portion of each caudal fin using the Kaneka Easy DNA extraction kit version 2 (Kaneka, Hyogo, Japan) or the DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany). Total genomic DNA was

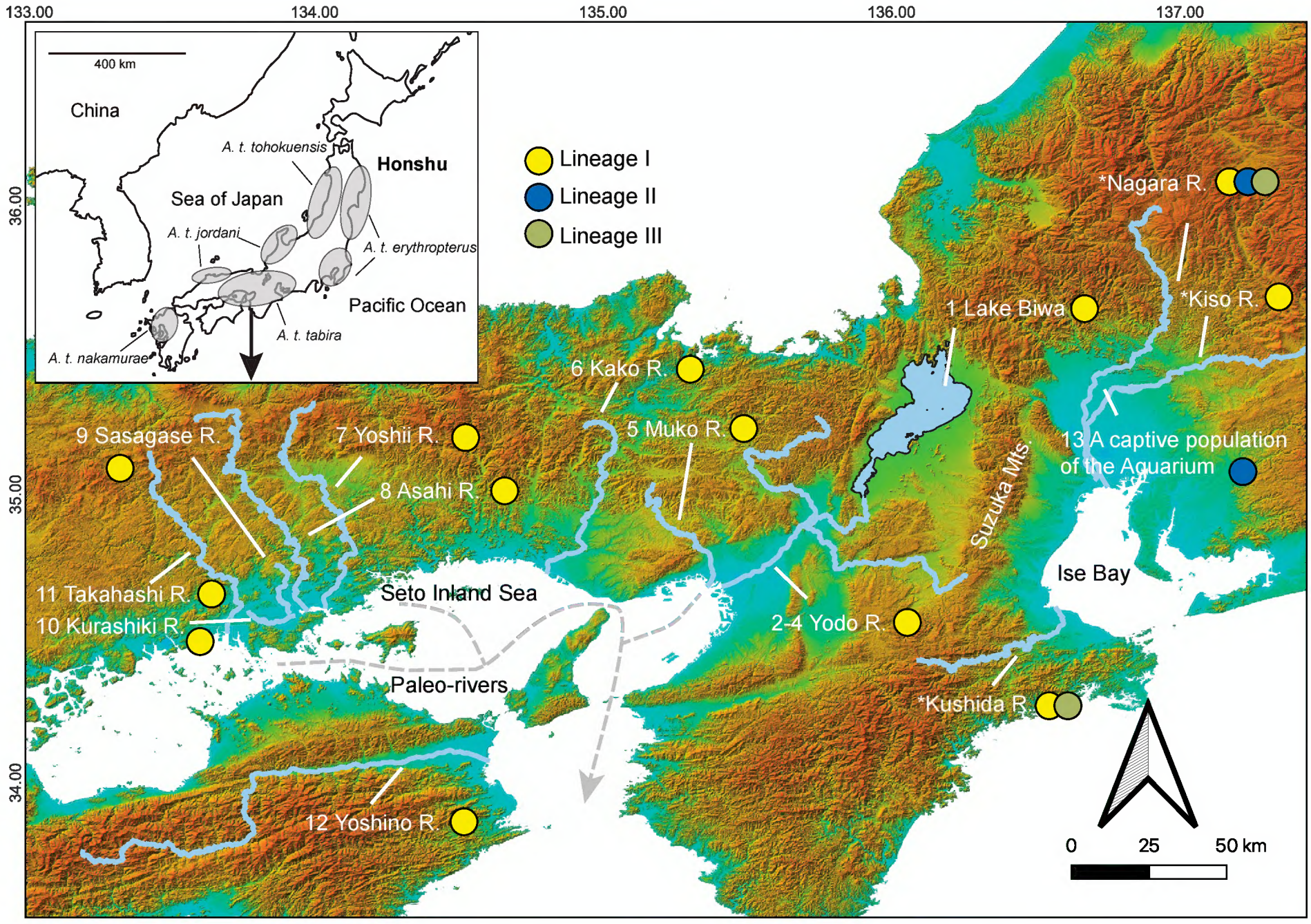

Figure 1. Sampling localities of *Acheilognathus tabira tabira*. The asterisks indicate localities used by Kitamura et al. (2012), Umemura et al. (2012), and Kitamura and Uchiyama (2020). Circles show mtDNA lineages (see Fig. 2). Gray areas represent the estimated natural distribution area of the five subspecies of *A. tabira* based on Kitamura et al. (2012). This altitude map was used with permission from the Geospatial Information Authority of Japan (https://maps.gsi.go.jp/) and digital national and information of Ministry of Land, Infrastructure, Transport and Tourism (https://nlftp.mlit.go.jp).

used to amplify DNA fragments using polymerase chain reaction (PCR). For PCR, the following forward primer was used: L14690-Cb-AH, 5'-GGT CAT AAT TCT TGC TCG GA-3' (Kitanishi et al. 2016), and for the reverse primers H15913-Thr-AH, 5'-CCG ATC TTC GGA TTA CAA GAC CG-3' (Kitanishi et al. 2016), or Cytb-Rev, 5'-GAT CTT CGG ATT ACA AGA CC-3' (Hashiguchi et al. 2006). The sequencing protocol was previously described by Ito et al. (2020). All sequences were deposited in the DNA Data Bank of Japan (DDBJ), European Nucleotide Archive (EMBL), and GenBank databases under the accession numbers LC775317–LC775352.

Sequence and phylogenetic analyses

Multiple alignments of nucleotide sequences were performed using MUSCLE (Edgar 2004). For the phylogenetic analysis, nucleotide sequence data for *A. t. tabira* were obtained from DDBJ, EMBL, and GenBank (AB620138, AB620141, AB620150, AB620159, AB759881–AB759890, and LC578851, Kitamura et al. 2012; Umemura et al. 2012; Ito et al. 2021; Table 1). In addition, we used *Acheilognathus tabira jordani* Arai, Fujikawa & Nagata, 2007, a sister group of *A. t. tabira*, as outgroups (AB620149, and AB620156, Kitamura et al. 2012).

Table 1. Sampling location numbers and names and GenBank accession numbers of samples.

Species name	Collection location	Accession No.	Haplotypes	Reference
Acheilognathus tabira tabira	Lake Biwa, Shiga, Japan	AB620138	–	Kitamura et al. 2012
A.t. tabira	Harai River, Mie, Japan	AB620141	–	Kitamura et al. 2012
A.t. tabira	Yoshii R., Okayama, Japan	AB620150	–	Kitamura et al. 2012
A.t. tabira	Kizu R., Kyoto, Japan	AB620159	–	Kitamura et al. 2012
A.t. tabira	Kiso R., Gifu, Japan	AB759881	–	Umemura et al. 2012
A.t. tabira	Kiso R., Gifu, Japan	AB759882	–	Umemura et al. 2012
A.t. tabira	Kiso R., Gifu, Japan	AB759883	–	Umemura et al. 2012
A.t. tabira	Kiso R., Gifu, Japan	AB759884	–	Umemura et al. 2012
A.t. tabira	Kiso R., Gifu, Japan	AB759885	–	Umemura et al. 2012
A.t. tabira	Nagara R., Gifu, Japan	AB759886	–	Umemura et al. 2012
A.t. tabira	Nagara R., Gifu, Japan	AB759887	–	Umemura et al. 2012
A.t. tabira	Nagara R., Gifu, Japan	AB759888	–	Umemura et al. 2012
A.t. tabira	Nagara R., Gifu, Japan	AB759889	–	Umemura et al. 2012
A.t. tabira	Nagara R., Gifu, Japan	AB759890	–	Umemura et al. 2012
A.t. tabira	Northern district, Mie, Japan	LC578851	–	Ito et al. 2021
A.t. tabira	Lake Biwa, Shiga, Japan	LC775317	T1	This study
A.t. tabira	Lake Biwa, Shiga, Japan	LC775318	T2	This study
A.t. tabira	Lake Biwa, Shiga, Japan, etc	LC775319	T3	This study
A.t. tabira	Lake Biwa, Shiga, Japan, etc	LC775320	T4	This study
A.t. tabira	Yodo R., Kyoto, Japan	LC775321	T5	This study
A.t. tabira	Yodo R., Kyoto, Japan	LC775322	T6	This study
A.t. tabira	Yodo R., Kyoto, Japan	LC775323	T7	This study
A.t. tabira	Yodo R., Kyoto, Japan	LC775324	T8	This study
A.t. tabira	Yodo R., Kyoto, Japan	LC775325	T9	This study
A.t. tabira	Yodo R., Kyoto, Japan	LC775326	T10	This study
A.t. tabira	Yodo R., Kyoto, Japan, etc	LC775327	T11	This study
A.t. tabira	Yodo R., Kyoto, Japan, etc	LC775328	T12	This study
A.t. tabira	Muko R., Hyogo, Japan	LC775329	T13	This study
A.t. tabira	Muko R., Hyogo, Japan	LC775330	T14	This study
A.t. tabira	Muko R., Hyogo, Japan	LC775331	T15	This study
A.t. tabira	Muko R., Hyogo, Japan	LC775332	T16	This study
A.t. tabira	Muko R., Hyogo, Japan	LC775333	T17	This study
A.t. tabira	Muko R., Hyogo, Japan	LC775334	T18	This study
A.t. tabira	Muko R., Hyogo, Japan	LC775335	T19	This study
A.t. tabira	Muko R., Hyogo, Japan	LC775336	T20	This study
A.t. tabira	Kako R., Hyogo, Japan	LC775337	T21	This study
A.t. tabira	Kako R., Hyogo, Japan	LC775338	T22	This study
A.t. tabira	Kako R., Hyogo, Japan, etc	LC775339	T23	This study
A.t. tabira	Yoshii R., Okayama, Japan, etc	LC775340	T24	This study
A.t. tabira	Yoshii R., Okayama, Japan	LC775341	T25	This study
A.t. tabira	Yoshii R., Okayama, Japan	LC775342	T26	This study
A.t. tabira	Yoshii R., Okayama, Japan, etc	LC775343	T27	This study
A.t. tabira	Yoshii R., Okayama, Japan	LC775344	T28	This study
A.t. tabira	Yoshii R., Okayama, Japan	LC775345	T29	This study
A.t. tabira	Asahi R., Okayama, Japan	LC775346	T30	This study
A.t. tabira	Sasagase R., Okayama, Japan	LC775347	T31	This study
A.t. tabira	Sasagase R., Okayama, Japan	LC775348	T32	This study
A.t. tabira	Sasagase R., Okayama, Japan	LC775349	T33	This study
A.t. tabira	Sasagase R., Okayama, Japan	LC775350	T34	This study
A.t. tabira	Yodo R., Kyoto, Japan	LC775351	T35	This study
A.t. tabira	Gifu R., Japan	LC775352	T36	This study
A.t. jordani	Oohara R., Shimane, Japan	AB620149	–	Kitamura et al. 2012
A.t. jordani	Kuzuryu R., Fukui, Japan	AB620156	–	Kitamura et al. 2012

Phylogenetic analyses were conducted using maximum likelihood (ML) and Bayesian inference (BI) methods. Search for the best evolutionary model each partition and ML analyses were performed using IQ-TREE 2.2.2.6 (Minh et al. 2020), with the TIM2 + F + I, K2P + I, and F81 + F models for the first, second, and third codon positions as selected by ModelFinder (Chernomor et al. 2016; Kalyaanamoorthy et al. 2017) based on the Bayesian information criterion (BIC). The reliability of each internal branch was evaluated using Shimodaira-Hasegawa-like approximate likelihood ratio test (SH-aLRT, Guindon et al. 2010) and ultrafast bootstrap (UFBoot, Hoang et al. 2018) and with 1000 replicates. In this study, according to the manual, support values of 80% or higher for SH-aLRT and 95% or higher for UFBoot were considered as high support values. BI analyses were performed using MrBayes v3.2.7a (Ronquist et al. 2012), with the HKY + I, K80 + I + G4, F81 models for the first, second, and third codon positions as selected by ModelTest-NG (Darriba et al. 2017) based on the BIC. The MCMC analyses were performed using the following settings: ngen = 10000000, sample freq = 100, and burnin = 25000. Statistical parsimony networks were constructed using TCS v1.2.1 (Clement et al. 2000). In this study, the Bayesian posterior probability (PP) of 80% or higher was considered to indicate high support values.

Genetic structure analyses

In populations of the Kinki-Sanyo region, we calculated genetic differentiation, estimated by genetic differentiation coefficient (Φ_{st}; Excoffier et al. 1992) using Arlequin version 3.5 (Excoffier and Lischer 2010). Critical significance levels for multiple testing were corrected using the sequential Bonferroni procedure (Rice 1989). In addition, we identified groups of populations using the spatial analysis of molecular variance version 2.0 (SAMOVA 2.0) (Dupanloup et al. 2002). To detect the number K of groups with the largest F_{ct} value, K was user-defined between 2 and 8, and 100 independent simulated annealing processes were performed in each run. However, collection sites with two or fewer individuals (the Kurashiki and Takahashi River systems) were excluded from the Φ_{st} and SAMOVA.

Estimation of divergence time

The divergence times of intraspecific lineage of Acheilognathus *tabira tabira* were estimated using BEAST ver. 2.7.6 (Bouckaert et al. 2019). We used sequences of *A. t. jordani*, *A. cyanostigma* Jordan & Fowler, 1903, and *Opsariichthys platypus* (Temminck & Schlegel, 1846) as outgroups. We adopted the estimated clock rate of cytb (0.76%, Zardoya and Doadrio 1999) that has been applied to the Acheilognathinae (Tominaga et al. 2020; Miyake et al. 2021). Divergence times were calibrated using the first appearance in the fossil record of Acheilognathinae from the early Miocene (ca. 20 Mya) found in Japan (Yasuno 1984). Constraints of the fossil were specified as a log-normal distribution, ranging from 16.5–23.0 Mya in the 95% range. Estimation was carried out using an optimised relaxed clock and applied the substitution model HKY+I selected by the BIC in ModelTest-NG. MCMC chains were run for 50000000 generations and sampled every 1000 generations, with the exclusion of the first

5000000 generations as burn-in. The convergence of MCMC was checked by calculating ESS values (> 200) using Tracer ver. 1.7.2 (Rambaut et al. 2018). TreeAnnotator ver. 2.7.6 in the BEAST package was used to obtain a maximum credibility tree with the annotation of average node ages and 95% highest posterior density (HPD) interval. The phylogenetic tree was visualized with FigTree ver. 1.4.4 (Rambaut 2018).

Results

Genetic structure

We sequenced 1069-bp mtDNA cytb gene nucleotide fragments from 140 individuals of *A. t. tabira* collected from 10 river systems and the captive population. As a result, 36 haplotypes were detected (T1–36), 5 of which [T1: AB759882 (Kiso River system), T3: AB759884 (Kiso R.), T4: AB620138, AB620159, and LC578851 (Lake Biwa, Kizu R., Kiso R., and northern Mie Prefecture), T23: AB759887 (Nagara R.), and T36: AB759888 (Nagara R.)] had been detected in previous studies (Kitamura et al. 2012; Umemura et al. 2012; Ito et al. 2021).

The topologies of the ML and BI phylogenetic trees were partially different (Fig. 2, Suppl. material 2). In the ML tree, Lineage I and III were supported as monophyletic with high values, respectively (SH-aLRT > 94%, UFBoot > 96%). The monophyletic clade of Lineage I and III was supported with high values (88.5%) by SH-aLRT, however with slightly lower value (88%) by UFBoot. In the BI tree, the monophyletic clade of Lineage I was supported by PP with a low value (0.54), while Lineage III was supported with a high value (0.99). The monophyletic clade of Lineage I and II was not supported, whereas the monophyletic clade of Lineage II and III was supported, albeit with a lower value (0.69). In addition, Lineage III was further subdivided into two sub-lineages: Sub-lineages III-i and ii in the ML and BI trees (SH-aLRT = 87.4%, UFBoot = 95%, and PP = 0.96, Fig. 2).

In Lineage I, Haplotypes T1–12 and T35 were mainly detected in Lake Biwa and the Yodo River system (Loc. 1–4), four of which (T3, T4, T11, and T12) were detected in the Yoshino River system (Loc. 12). Haplotypes L13–20 were detected only in the Muko River system (Loc. 5). Haplotypes T21 and T22 were only detected in the Kako River system (Loc. 6). Haplotype T23 was detected in the Kako, Yoshii Asahi, Sasagase, and Takahashi River systems (Loc. 6–9, and 11). Haplotypes T24–34 were detected in the Yoshii Asahi, Sasagase, Kurashiki, and Takahashi River systems (Loc. 7–11). Haplotype T36 in Lineage II was detected only in a captive Gifu World Freshwater Aquarium population collected from an unknown river system in Gifu Prefecture (Loc. 13).

In the statistical parsimony network, *A. t. tabira* exhibits a bottleneck pattern (Fig. 2). Lineage I was represented by a star-like pattern centered on the ancestral haplotype T4 (Fig. 2).

The results of the pairwise Φ_{st} among the local populations are shown in Table 2. The Φ_{st} values of Yodo1 and Muko populations showed significant genetic differentiation between populations, excluding Yodo3 and Biwa populations, respectively (Φ_{st} 0.235–392, 0.269–0.415; $P < 0.05$). The Φ_{st} value of the Yoshino population also showed significant genetic differentiation between Kako, Yoshii, Asahi, and Sasagase populations (Φ_{st} 0.191–0.301: $P < 0.05$).

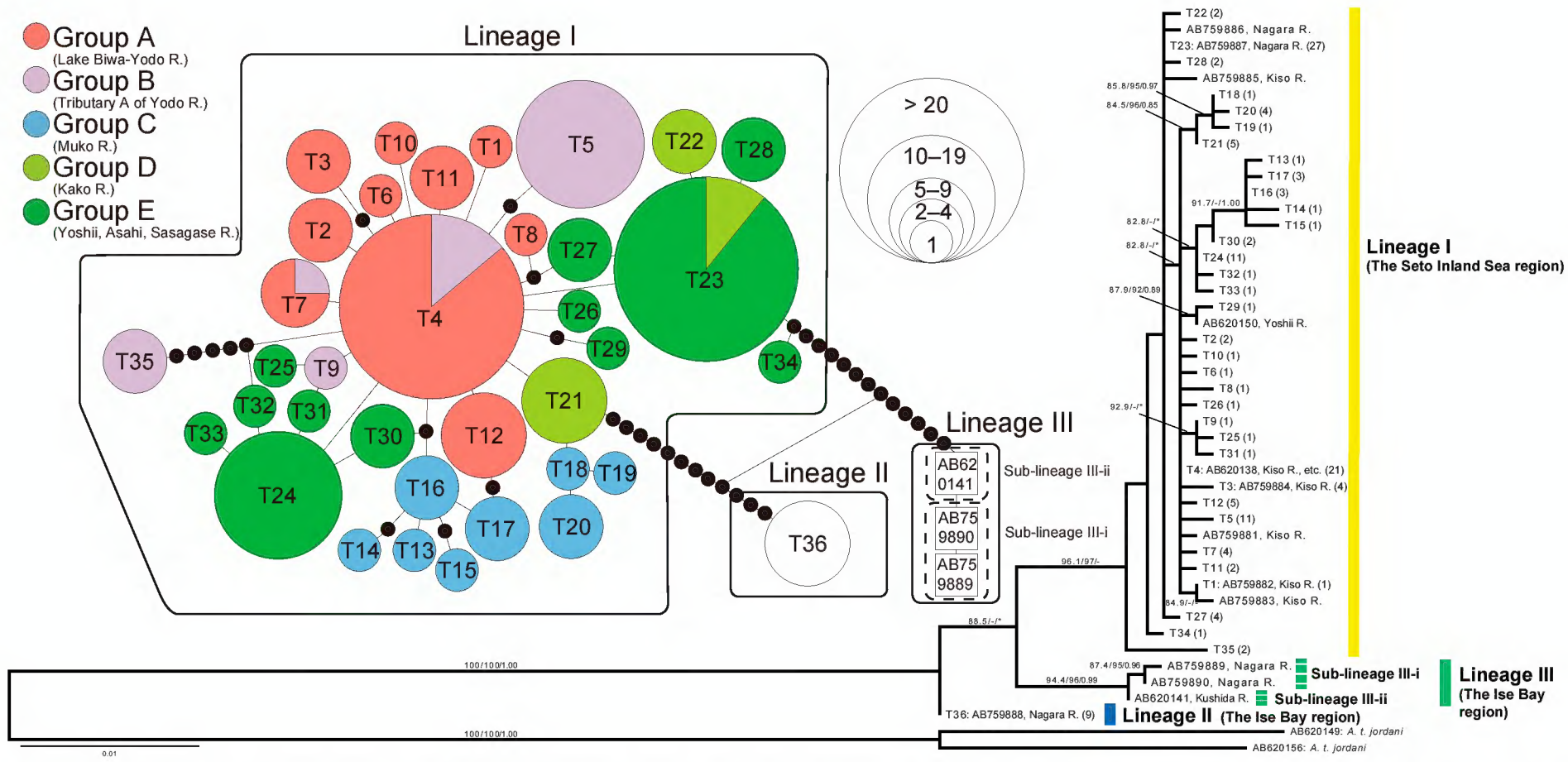

Figure 2. Maximum likelihood (ML) tree of the 1069-bp cytochrome *b* gene sequences of *Acheilognathus tabira tabira* individuals from the Seto Inland Sea and Ise Bay regions. Numbers at nodes indicate Shimodaira-Hasegawa-like approximate likelihood ratio test values (left), ultrafast bootstrap values (middle) in the ML tree, and Bayesian posterior probabilities (right) in Bayesian inference tree. Each value is indicated when it exceeds 80%, 95%, and 0.80. Numbers in *parentheses* indicate the number of specimens. The parentheses after each Lineage name indicate the natural distribution area. The statistical parsimony network of *A. t. tabira* is shown to the left of the tree. Pie charts of Lineage I indicate the relative frequencies of haplotypes of the five groups defined by SAMOVA.

Table 2. Pairwise Φ_{st} among local populations of *Acheilognathus tabira tabira* collected from the Seto Inland Sea region.

Site no.	Collection site	Group	1	2	3	4	5	6	7	8	9	12
1	Lake Biwa	A										
2	Yodo R. 1	B	0.235*									
3	Yodo R. 2	A	0.042	0.237*								
4	Yodo R. 3	A	0.019	0.236	-0.004							
5	Muko R.	C	0.266	0.358**	0.280*	0.261**						
6	Kako R.	D	0.271	0.339**	0.298	0.256**	0.281*					
7	Yoshii R.	E	0.159	0.301**	0.145	0.164	0.330**	0.135				
8	Asahi R.	E	0.282*	0.359**	0.297*	0.269**	0.349**	0.241	-0.006			
9	Sasagase R.	E	0.331**	0.392**	0.340**	0.321**	0.415**	0.186	0.018	0.072		
12	Yoshino R.	A	0.033	0.261**	0.093	-0.047	0.269**	0.289**	0.191*	0.301**	0.349**	

*$P < 0.05$; **$P < 0.001$

In addition, the Φ_{st} values of the Asahi and Sasagase populations showed significant genetic differentiation between Biwa, Yodo2, and Yodo3 populations (Φ_{st} 0.269–0.392: $P < 0.05$), and Kako population showed significant genetic differences from Yodo3 population (Φ_{st} 0.281–0.339, $P < 0.05$).

The results of the population group estimation using SAMOVA are shown in Table 3 and Suppl. material 3. The highest F_{ct} value (0.32166; $P < 0.001$) was obtained when the 10 populations were divided into $K = 5$ groups: Group A (Lake Biwa-Yodo River and Yoshino River: Loc. 1, 3, 4, and 12), Group B (tributary A of Yodo River: Loc. 2), Group C (Muko R. Loc. 5), Group D (Kako R.: Loc. 6) and Group E

Table 3. Fixation indicating corresponding groups of populations inferred by spatial analysis of molecular variance (SAMOVA).

Number of groups (*K*)	Group composition	F_{sc}	F_{st}	F_{ct}
2	"Biwa"+"Yodo1"+"Yodo2"+"Yodo3"+"Kako"+"Yoshii"+"Asahi"+"Aasagase"+"Yoshino" "Muko"	0.21769***	0.42786***	0.26865**
3	"Biwa"+"Yodo2"+"Yodo3"+"Kako"+"Yoshii"+"Asahi"+"Sasagase"+"Yoshino" "Muko" "Yodo1"	0.14052***	0.38805***	0.288*
4	"Biwa"+"Yodo2"+"Yodo3"+"Yoshino" "Kako"+"Yoshii"+"Asahi"+"Sasagase" "Muko" "Yodo1"	0.0344**	0.33127***	0.30745***
5	"Biwa"+"Yodo2"+"Yodo3"+"Yoshino" "Yoshii"+"Asahi"+"Sasagase" "Muko" "Yodo1" "Kako"	-0.00438	0.31869***	0.32166***
6	"Biwa"+"Yodo2"+"Yodo3"+"Yoshino" "Yoshii"+"Asahi" "Muko" "Yodo1" "Kako" "Sasagase"	-0.02137	0.30139***	0.31601***
7	"Yodo2"+"Yodo3"+"Yoshino" "Yoshii"+"Asahi" "Muko" "Yodo1" "Kako" "Sasagase" "Biwa"	-0.02246	0.29394***	0.30945**
8	"Yodo2"+"Yodo3"+"Yoshino" "Muko" "Yodo1" "Yoshii" "Kako" "Sasagase" "Biwa" "Asahi"	-0.03234	0.2872***	0.30953*

$P < 0.05$*, $P < 0.01$**, $P < 0.001$***

(Yoshii, Asahi, and Sasagase Rs: Loc. 7–9), and explained 32.17% of the variation among groups ($P < 0.001$), -0.3% of the variation among populations within groups ($P > 0.1$), and 68.13% of the variation within populations ($P < 0.001$).

Divergence time

We showed the divergence times of the three lineages of *A. t. tabira* in Fig. 3. In this tree, the exclusivity of Lineage I was supported (1.00), similar to the ML and BI trees described above, whereas the exclusivity of Lineages II and

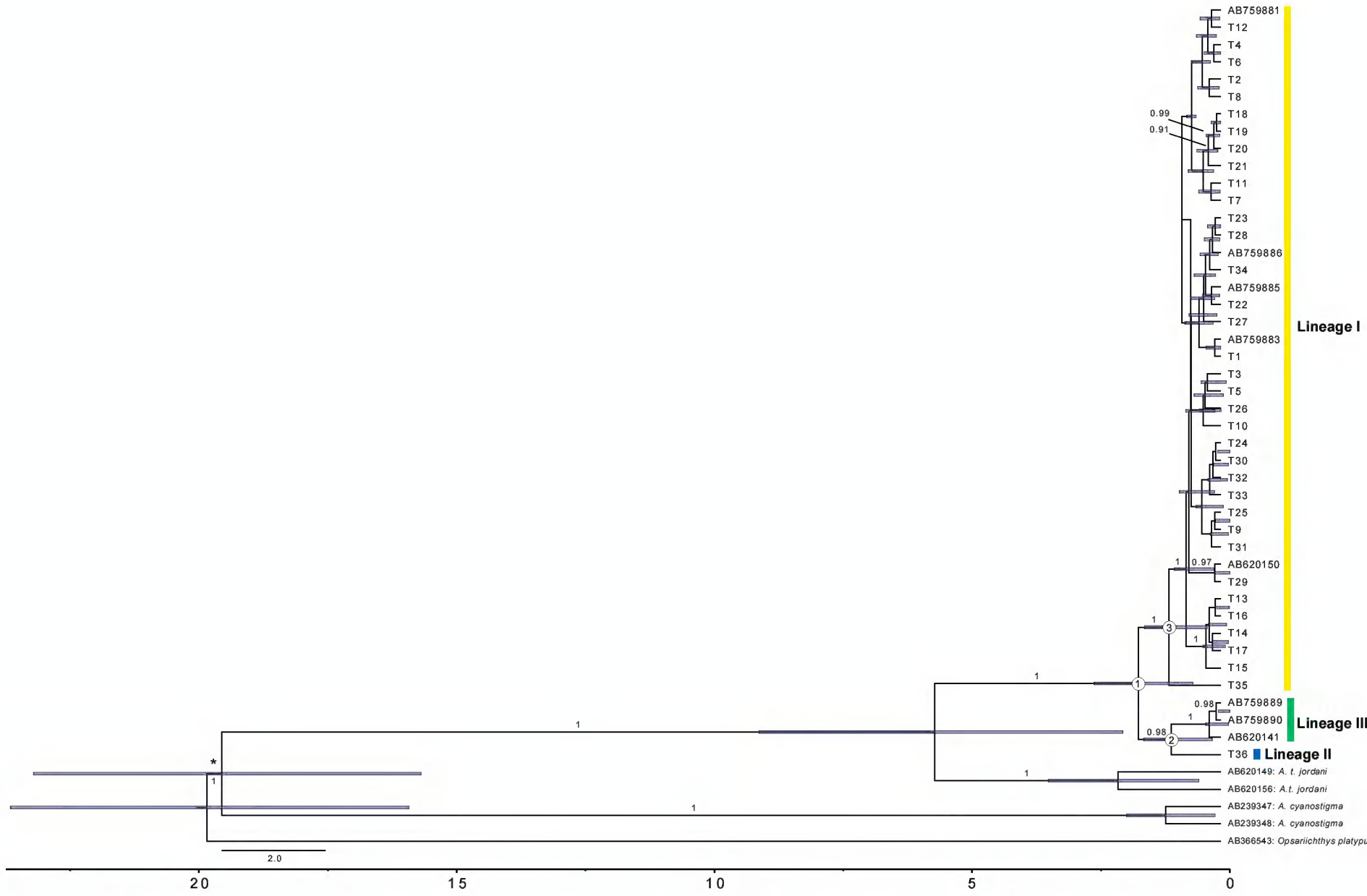

Figure 3. Divergence time estimation by Bayesian inference tree of the 1069-bp cytochrome *b* gene sequences of *Acheilognathus tabira tabira* and outgroups. The blue rectangular bars on the nodes indicate the 95% highest probability density. Bayesian posterior probabilities are indicated at nodes, with values exceeding 0.90 shown. The node marked with an asterisk indicates the calibration point based on fossil record for the Acheilognathinae. Nodes with circled numbers are referenced in the text.

III was supported (0.98), similar to the topology of the BI tree. The estimated divergence time between Lineage I and II+III was approximately 1.53 Mya (95% HPD, 0.71–2.64 Mya; Fig. 3, node 1), while that between Lineage II and III was approximately 0.91 Mya (0.34–1.68 Mya; node 2). The time of the most recent common ancestor (tMRCA) of Lineage I was estimated approximately 0.96 Mya (0.44–1.66 Mya; Fig. 3, node 3).

Discussion

Phylogeographic and genetic population structure patterns

We estimated the phylogenetic tree of *A. t. tabira* based on the sequence of the cytochrome *b* region of the mtDNA, and in the samples used in the present study, three lineages (Lineages I, II, and III) were identified primarily based on the ML tree. The results were similar to those of a previous study (Umemura et al. 2012), where Lineage I to III were referred to as the Kinki-Sanyo Group, Nobi Plain Group I, and Nobi Plain Group II, respectively. The populations newly analyzed in the present study (Muko, Kako, Sasagase, Takahashi, Kurashiki, and Asahikawa River systems) were all included in Lineage I, and the captive Gifu World Freshwater Aquarium population collected from Gifu Prefecture was included in Lineage II. In a previous study, a haplotype of Lineage II was detected

only in an individual collected from a tributary of the Nagara River system in the previous study (Umemura et al. 2012). Lineages I (non-native lineage) and III (native lineage) were identified in this tributary; therefore, it was unclear whether Lineage II was native to the Ise Bay region or non-native to the Seto Inland Sea region (Umemura et al. 2012). In the present study, the haplotype belonging to Lineage II was not identified in any water system in the Seto Inland Sea region; therefore, the natural distribution area of Lineage II was considered to be the Ise Bay region.

The populations of many freshwater fishes [e.g., *Sarcocheilichthys variegatus variegatus* (Temminck & Schlegel, 1846) and *Opsariichthys platypus*] in the Ise Bay region are thought to have been divided from the populations of the Seto Inland Sea region by the uplift of the Suzuka Mountains approximately one million years ago (Mya) (Watanabe et al. 2017). The genetic lineages of populations in many freshwater fishes in each region of Ise Bay and the Seto Inland Sea are generally exclusive (e.g., *S. v. variegatus*, Komiya et al. 2014; *O. platypus*, Kitanishi et al. 2016; *Pseudogobio esocinus* (Temminck & Schlegel, 1846) and *P. agathonectris* Tominaga & Kawase, 2019, Tominaga et al. 2016; T*anakia lanceolata* (Temminck & Schlegel, 1846), Tominaga et al. 2020). However, in the case of *A. t. tabira*, the exclusivity of the two native lineages in the Ise Bay region (lineages II and III) was not supported in the ML tree and was supported with low values in the BI tree. In the ML tree, these two lineages were shown as paraphyletic groups, and each branch was highly supported. Furthermore, the results of the statistical parsimony network also indicated that lineages II and III were closely related to different haplotypes of Lineage I. On the other hand, the BI tree does not have the same topology as the ML tree. Divergence time estimates suggest that the supported topology is similar to that of the BI tree, with Lineages II and III diverging from Lineage I approximately 1.53 Mya, and Lineages II and III diverging approximately 0.91 Mya. However, due to the varying exclusivity of Lineages II and III across different phylogenetic trees, accepting this divergence time estimation as it is would be risky. And more, the original distribution range of Lineage II and III is unclear, as *A. t. tabira* in the Ise Bay region has already become extinct in many river systems (Mukai 2019). If DNA analysis of specimens collected decades ago and stored in museums becomes possible, it will be possible to verify this issue. To consider the divergence order among the three lineages, further studies using longer sequences, such as mitogenomes, including sequence data from historical specimens, are necessary to re-estimate divergence times.

In Lineage I, which was detected only in the Seto Inland Sea region, genetic differentiation has not been recognized in previous studies because of the small number of sampling sites and individuals (Kitamura et al. 2012). In the present study, we greatly increased the number of sampling sites and individuals; as a result, five genetic groups (A–E) were distinguished within Lineage I using SAMOVA. These five groups comprised adjacent river systems, indicating that the genetic population of *A. t. tabira* within the Seto Inland Sea region was genetically differentiated into narrow regions. The river systems flowing into the Seto Inland Sea are thought to have connected as a single paleo-river system during the glacial periods of the Pleistocene (ca. 0.01–2.5 Mya) and were isolated during the interglacial periods (Kuwashiro 1959; Ota et al. 2004). Additionally, the uplift of the mountains areas surrounding the Seto Inland

Sea is thought to have become active since the Pleistocene (Ota et al. 2004), making the migration of freshwater fish between river systems difficult during this period. The tMRCA of Lineage I is estimated to be 0.96 Mya (95% HPD, 0.44–1.66 Mya), overlapping with the periods of connection and isolation of the paleo-river systems and the uplift of mountains around the Seto Inland Sea. Therefore, the isolation factors between the regional groups are suggested to be related to the paleo-river systems and active uplift of mountains that occurred during the Pleistocene. Genetic differentiation in the same period due to similar factors in the Seto Inland Sea region has also been suggested in *P. esocinus* (Tominaga et al. 2016).

A unique genetic group (Group D) was identified in the Kako River system. However, Φ_{st} showed no significant genetic differentiation ($P > 0.05$) between the Kako River system and the other three river systems (Yoshii, Asahi, and Sasagase) included in Group E. SAMOVA results indicated that most of the genetic variation in this subspecies was within populations (68.13%) and that differentiation among groups was relatively small (32.17%). Genome-wide analysis of nuclear DNA may be useful for more detailed elucidation of the genetic population structure of *A. t. tabira*.

Artificially introduced populations

Populations collected from the Lake Biwa-Yodo and Yoshino River systems were included in Group A. In addition, four haplotypes detected in the Yoshino River system were similar to those in the Lake Biwa-Yodo River system. This study demonstrates that populations in the Seto Inland Sea region are genetically differentiated by localized areas. The reason for this is thought to be the same as with other species: the disappearance of the paleo-river system and isolation due to the uplift of mountains. Therefore, it is unlikely that the population in the Yoshino River system has the same haplotype as the population in the Lake Biwa–Yodo River system, which is across the Seto Inland Sea. In the Yoshino River system, non-native freshwater fishes [e.g., *Acheilognathus cyanostigma* and *Acheilognathus rhombeus* (Temminck & Schlegel, 1846)] were estimated to have been artificially introduced from Lake Biwa (Hosoya 2019; Miyake et al. 2021). Ministry of the Environment of Japan (2015) also indicated that *A. t. tabira* populations were artificially introduced into the Yoshino River system. Therefore, the finding that only haplotypes of the Yoshino River system are common to those of the Lake Biwa-Yodo River system supports the hypothesis that the Yoshino River system population was artificially introduced from the Lake Biwa-Yodo River system.

Conservation

The captive population of the Gifu World Freshwater Aquarium was identified as Lineage II, which is thought to be native to the Ise Bay region. Non-native populations belonging to Lineage I have been artificially introduced into all habitats of native populations in the Ise Bay region (Umemura et al. 2012; Kitamura and Uchiyama 2020). Therefore, the captive population of the Gifu World Freshwater Aquarium may be a native population of the Ise Bay region that has not undergone genetic introgression. However, to confirm that the population

is not genetically introgressed, examining the possibility of hybridization with Lineage I using nuclear DNA is necessary.

Conservation units need to focus on levels below species (Moritz 1994, Frankham et al. 2010). Evolutionarily significant units (ESUs) refer to phylogenetically unique intraspecific population groups, established by factors such as mitochondrial DNA monophyly (Moritz 1994). In the case of *A. t. tabira*, the exclusivity of Lineages I–III was supported; thus, we propose to designate them as ESUs.

Furthermore, Management Units (MUs) are established based on allele frequencies among populations (Moritz 1994, Frankham et al. 2010). In the case of *A. t. tabira*, Groups A–E found within Lineage I were significantly genetically differentiated by SAMOVA. Therefore, we propose that these five groups be conserved as MUs of *A. t. tabira* in the Seto Inland Sea region. However, it has been proposed that adaptive traits should also be taken into account in the establishment of ESUs and MUs (Crandall et al. 2000). In the future, in order to establish better conservation units, it is necessary to study adaptive traits among populations of *A. t. tabira*.

Acknowledgments

We express sincere thanks to Mr. Koki Ikeya, Ms. Chikako Horie, Mr. Kosei Nishikawa, and Mr. Jumpei Hamachi for their assistance with obtaining the specimens, and to Mr. Ken-ichi Setsuda for registering the specimens. Furthermore, we extend our appreciation to the Division of Genomics Research, Dr. Hiroki Yamanaka, and the Life Science Research Center, Gifu University, for their help with DNA analysis.

Additional information

Conflict of interest

The authors have declared that no competing interests exist.

Ethical statement

No ethical statement was reported.

Funding

This study was supported by JSPS KAKENHI (22K14908).

Author contributions

Gen Ito: Conceptualization, Data curation, Formal Analysis, Funding acquisition, and Writing – original draft. Naoto Koyama, Ryota Noguchi, Ryoichi Tabata, Seigo Kawase, and Jyun-ichi Kitamura: Investigation, Resources, and Writing – review & editing. Yasunori Koya: Supervision, Funding acquisition, and Writing – review & editing.

Author ORCIDs

Gen Ito https://orcid.org/0000-0002-9781-7206

Data availability

All of the data that support the findings of this study are available in the main text or Supplementary Information.

References

Arai R, Fujikawa H, Nagata Y (2007) Four new species of *Acheilognathus* bitterlings (Cyprinidae: Acheilognathinae) from Japan. Bulletin of the National Museum of Nature and Science, Series A (Supplement 1): 1–28.

Avise JC (2000) Phylogeography: the history and formation of species. Harvard University Press, Cambridge, 464 pp. https://doi.org/10.2307/j.ctv1nzfgj7

Bouckaert R, Vaughan TG, Barido-Sottani J, Duchêne S, Fourment M, Gavryushkina A, Heled J, Jones G, Kühnert D, De Maio N, Matschiner M, Mendes FK, Müller NF, Ogilvie HA, du Plessis L, Popinga A, Rambaut A, Rasmussen D, Siveroni I, Suchard MA, Wu CH, Xie D, Zhang C, Stadler T, Drummond AJ (2019) BEAST 2.5: An advanced software platform for Bayesian evolutionary analysis. PLoS Computational Biology 15(4): e1006650. https://doi.org/10.1371/journal.pcbi.1006650

Chernomor O, von Haeseler A, Minh BQ (2016) Terrace aware data structure for phylogenomic inference from supermatrices. Systematic Biology 65(6): 997–1008. https://doi.org/10.1093/sysbio/syw037

Clement M, Posada D, Crandall K (2000) TCS: A computer program to estimate gene genealogies. Molecular Ecology 9(10): 1657–1660. https://doi.org/10.1046/j.1365-294x.2000.01020.x

Crandall KA, Bininda-Emonds ORP, Mace GM, Wayne RK (2000) Considering evolutionary processes in conservation biology. Trends in Ecology & Evolution 15(7): 290–295. https://doi.org/10.1016/S0169-5347(00)01876-0

Darriba D, Posada D, Stamatakis A (2017) ModelTest-NG: Best-fit evolutionary model selection. https://github.com/ddarriba/modeltest/ [Accessed 9 September 2022]

Dupanloup I, Schneider S, Excoffier L (2002) A simulated annealing approach to define the genetic structure of populations. Molecular Ecology 11(12): 2571–2581. https://doi.org/10.1046/j.1365-294X.2002.01650.x

Edgar RC (2004) MUSCLE: Multiple sequence alignment with high accuracy and high throughput. Nucleic Acids Research 32(5): 1792–1797. https://doi.org/10.1093/nar/gkh340

Excoffier L, Lischer HE (2010) Arlequin suite ver 3.5: A new series of programs to perform population genetics analyses under Linux and Windows. Molecular Ecology Resources 10(3): 564–567. https://doi.org/10.1111/j.1755-0998.2010.02847.x

Excoffier L, Smouse PE, Quattro JM (1992) Analysis of molecular variance inferred from metric distances among DNA haplotypes: Application to human mitochondrial DNA restriction data. Genetics 131(2): 479–491. https://doi.org/10.1093/genetics/131.2.479

Frankham R, Ballou JD, Briscoe DA (2010) Introduction to conservation genetics. Second edition. Cambridge University Press, Cambridge, 618 pp.

Guindon S, Dufayard JF, Lefort V, Anisimova M, Hordijk W, Gascuel O (2010) New algorithms and methods to estimate maximum-likelihood phylogenies: Assessing the performance of PhyML 3.0. Systematic Biology 59(3): 307–321. https://doi.org/10.1093/sysbio/syq010

Hashiguchi Y, Kado T, Kimura S, Tachida H (2006) Comparative phylogeography of two bitterlings, *Tanakia lanceolata* and *T. limbata* (Teleostei, Cyprinidae), in Kyushu and adjacent districts of western Japan, based on mitochondrial DNA analysis. Zoological Science 23(4): 309–322. https://doi.org/10.2108/zsj.23.309

Hoang DT, Chernomor O, von Haeseler A, Minh BQ, Vinh LS (2018) UFBoot2: Improving the ultrafast bootstrap approximation. Molecular Biology and Evolution 35(2): 518–522. https://doi.org/10.1093/molbev/msx281

Hosoya K (2019) Freshwater fishes of Japan. Extended and revised edition, Yama-kei Publishers, Tokyo, 559 pp. [In Japanese]

Ito G, Koya Y (2022) Phylogeographic structure of an endemic lineage of the eight-barbel loach *Lefua echigonia* around the Suzuka and Yoro Mountains, central Honshu, Japan. Biogeography 24: 31–37. https://doi.org/10.11358/biogeo.24.39

Ito G, Koya Y, Kitanishi S, Horiike T, Mukai T (2019) Genetic population structure of the eight-barbel loach *Lefua echigonia* in the Ise Bay region, a single paleo-river basin in central Honshu, Japan. Ichthyological Research 66(3): 411–416. https://doi.org/10.1007/s10228-019-00683-z

Ito G, Koya Y, Kitanishi S, Horiike T, Mukai T (2020) Genetic population structure of *Cobitis minamorii tokaiensis*. Japanese Journal of Ichthyology 67: 41–50. https://doi.org/10.11369/jji.19-028 [In Japanese with English summary]

Ito G, Kitamura J, Noguchi R, Nagata N, Koya Y (2021) Records and genetic characteristics of exotic bitterling, *Acleilognathus tabira* subspp., from northern Mie Prefecture, Japan. Japanese Journal of Ichthyology 68: 47–52. https://doi.org/10.11369/jji.20-034 [In Japanese with English summary]

Ito G, Hata K, Kitamura J, Koya Y (2022) Records and genetic characteristics of the bitterling *Tanakia lanceolata* established in the Naruse River system, Miyagi Prefecture, Japan. Japanese Journal of Ichthyology 69: 57–62. [In Japanese with English summary] https://doi.org/10.11369/jji.20-24

Kalyaanamoorthy S, Minh BQ, Wong TKF, von Haeseler A, Jermiin LS (2017) ModelFinder: Fast model selection for accurate phylogenetic estimates. Nature Methods 14(6): 587–589. https://doi.org/10.1038/nmeth.4285

Kitagawa T, Yoshida M, Kashiwagi M, Okazaki T (2001) Population structure and local differentiation in the delicate loach, *Niwaella delicate*, as revealed by mitochondrial DNA and morphological analyses. Ichthyological Research 48(2): 127–135. https://doi.org/10.1007/s10228-001-8127-4

Kitamura J, Uchiyama R (2020) Bitterling fishes of Japan-natural history and culture. Yama-kei Publishers, Tokyo, 223 pp. [In Japanese with English summary]

Kitamura J, Nagata N, Nakajima J, Sota T (2012) Divergence of ovipositor length and egg shape in a brood parasitic bitterling fish through the use of different mussel hosts. Journal of Evolutionary Biology 25(3): 566–573. https://doi.org/10.1111/j.1420-9101.2011.02453.x

Kitanishi S, Hayakawa A, Takamura K, Nakajima J, Kawaguchi Y, Onikura N, Mukai T (2016) Phylogeography of *Opsariichthys platypus* in Japan based on mitochondrial DNA sequences. Ichthyological Research 63(4): 506–518. https://doi.org/10.1007/s10228-016-0522-y

Komiya T, Fujita-Yanagibayashi S, Watanabe K (2014) Multiple colonization of Lake Biwa by *Sarcocheilichthy*s fishes and their population history. Environmental Biology of Fishes 97(7): 741–755. https://doi.org/10.1007/s10641-013-0176-9

Kumagai M, Hagiwara T (2013) *Acheilognathus tabira erythropterus* and *A. t. tabira* collected from Aomori Prefecture, Japan. Izunuma-Uchinuma Wetland Researches 7: 17–22. [In Japanese with English summary] https://doi.org/10.20745/izu.7.0_17

Kuwashiro I (1959) Submarine topography of Japanese Inland Sea Setonaikai. Geographical Review of Japan 32(1): 24–34. https://doi.org/10.4157/grj.32.24 [In Japanese with English summary]

Machida H, Matsuda T, Umitsu M, Koizumi T (2006) Geomorphology of Chubu. Regional geomorphology of the Japanese islands, Vol 5. University of Tokyo Press, Tokyo, 385 pp. [In Japanese]

Matsuba H, Yoshimi S, Inoue M, Hata H (2014) Origin of *Tanakia limbata* in Ehime Prefecture indicated by phylogeographic analysis of mitochondrial cytochrome *b* gene sequences. Japanese Journal of Ichthyology 61: 89–96. https://doi.org/10.11369/jji.61.89 [In Japanese with English summary]

Minh BQ, Schmidt HA, Chernomor O, Schrempf D, Woodhams MD, von Haeseler A, Lanfear R (2020) IQ-TREE 2: New models and efficient methods for phylogenetic inference in the genomic era. Molecular Biology and Evolution 37(5): 1530–1534. https://doi.org/10.1093/molbev/msaa015

Ministry of the Environment of Japan (2015) Red data book 2014 – Threatened wildlife of Japan – volume 4, Pisces – Brackish and freshwater fishes. Gyosei Corporation, Tokyo, 414 pp. [In Japanese]

Ministry of the Environment of Japan (2020) The Ministry of Environment, Japan Red List. The Ministry of Environment, Japan, Tokyo. https://www.env.go.jp/press/files/jp/114457.pdf [Accessed 9 September 2022] [in Japanese]

Miyake T, Nakajima J, Onikura N, Ikemoto S, Iguchi K, Komaru A, Kawamura K (2011) The genetic status of two subspecies of *Rhodeus atremius*, an endangered bitterling in Japan. Conservation Genetics 12(2): 383–400. https://doi.org/10.1007/s10592-010-0146-0

Miyake T, Nakajima J, Umemura K, Onikura N, Ueda T, Smith C, Kawamura K (2021) Genetic diversification of the Kanehira bitterling *Acheilognathus rhombeus* inferred from mitochondrial DNA, with comments on the phylogenetic relationship with its sister species *Acheilognathus barbatulus*. Journal of Fish Biology 2021(5): 1–10. https://doi.org/10.1111/jfb.14876

Moritz C (1994) Defining 'Evolutionarily Significant Units' for conservation. Trends in Ecology & Evolution 9(10): 373–375. https://doi.org/10.1016/0169-5347(94)90057-4

Mukai T (2019) Fishes of Gifu, 2nd edition. Gifu Shimbun Gifu, Gifu, 223 pp. [In Japanese]

Nakagawa H, Seki S, Ishikawa T, Watanabe K (2016) Genetic population structure of the Japanese torrent catfish *Liobagrus reinii* (Amblycipitidae) inferred from mitochondrial cytochrome *b* variations. Ichthyological Research 63(3): 333–346. https://doi.org/10.1007/s10228-015-0503-6

Ota Y, Naruse T, Tanaka S, Okada A (2004) Geomorphology of Kinki, Chugoku and Shikoku. Regional geomorphology of the Japanese islands, Vol 6. University of Tokyo Press, Tokyo, 383 pp. [In Japanese]

Rambaut A (2018) FigTree v1.4.4. https://github.com/rambaut/figtree/releases [Accessed 1 June 2024]

Rambaut A, Drummond AJ, Xie D, Baele G, Suchard MA (2018) Posterior summarization in Bayesian phylogenetics using Tracer 1.7. Systematic Biology 67(5): 901–904. https://doi.org/10.1093/sysbio/syy032

Rice WR (1989) Analyzing tables of statistical test. Evolution; International Journal of Organic Evolution 43(1): 223–225. https://doi.org/10.2307/2409177

Ronquist F, Teslenko M, van der Mark P, Ayres DL, Darling A, Höhna S, Larget B, Liu L, Suchard MA, Huelsenbeck JP (2012) MyBayes 3.2: Efficient Bayesian phylogenetic inference and model choice across a large model space. Systematic Biology 61(3): 539–542. https://doi.org/10.1093/sysbio/sys029

Tominaga K, Nakajima J, Watanabe K (2016) Cryptic divergence and phylogeography of the pike gudgeon *Pseudogobio esocinus* (Teleostei: Cyprinidae): a comprehensive case of freshwater phylogeography in Japan. Ichthyological Research 63(1): 79–93. https://doi.org/10.1007/s10228-015-0478-3

Tominaga K, Nagata N, Kitamura J, Watanabe K, Sota T (2020) Phylogeography of the bitterling *Tanakia lanceolata* (Teleostei: Cyprinidae) in Japan inferred from mitochondrial cytochrome *b* gene sequences. Ichthyological Research 67(1): 105–116. https://doi.org/10.1007/s10228-019-00715-8

Uemura Y, Yoshimi S, Hata H (2018) Hybridization between two bitterling fish species in their sympatric range and a river where one species is native and the other is introduced. PLOS ONE 13(9): e0203423. https://doi.org/10.1371/journal.pone.0203423

Umemura K, Futamura R, Takagi M, Ikeya K, Mukai T (2012) Distribution of non-indigenous mitochondrial DNA lineage in the local populations of an endangered bitterling, *Acheilognathus tabira tabira*, in the Gifu Prefecture, Japan. Nihon Seibutsu Chiri Gakkai Kaiho 67: 169–174.

Watanabe K, Kawase S, Mukai T, Kakioka R, Miyazaki J, Hosoya K (2010) Population divergence of *Biwia zezera* (Cyprinidae: Gobioninae) and the discovery of a cryptic species, based on mitochondrial and nuclear DNA sequence analyses. Zoological Science 27(8): 647–655. https://doi.org/10.2108/zsj.27.647

Watanabe K, Mori S, Tanaka T, Kanagawa N, Itai T, Kitamura J, Suzuki N, Tominaga T, Kakioka R, Tabata R, Abe T, Tashiro Y, Hashimoto Y, Nakajima J, Onikura N (2014) Genetic population structure of *Hemigrammocypris rasborella* (Cyprinidae) inferred from mtDNA sequences. Ichthyological Research 61(4): 352–360. https://doi.org/10.1007/s10228-014-0406-y

Watanabe K, Tominaga K, Nakajima J, Kakioka R, Tabata R (2017) Japanese freshwater fishes: Biogeography and cryptic diversity. In: Motokawa M, Kajihara H (Eds) Species Diversity of Animal in Japan, Diversity and Commonality in Animals. Springer Japan, Tokyo, 183–227. https://doi.org/10.1007/978-4-431-56432-4_7

Yasuno K (1984) Fossil pharyngeal teeth of the Rhodeinae fish from the Miocene Katabira Formation of the Kani Group, Gifu Prefecture, Japan. Bulletin of Mizunami Fossil Museum 11: 101–105.

Zardoya R, Doadrio I (1999) Molecular evidence on the evolutionary and biogeographical patterns of European cyprinids. Journal of Molecular Evolution 49(2): 227–237. https://doi.org/10.1007/PL00006545

Supplementary material 1

List of collection sites for *Acheilognathus tabira tabira* and distribution of each haplotype across the 13 collection sites

Authors: Gen Ito, Naoto Koyama, Ryota Noguchi, Ryoichi Tabata, Seigo Kawase, Jyunichi Kitamura, Yasunori Koya

Data type: xlsx

Copyright notice: This dataset is made available under the Open Database License (http://opendatacommons.org/licenses/odbl/1.0/). The Open Database License (ODbL) is a license agreement intended to allow users to freely share, modify, and use this Dataset while maintaining this same freedom for others, provided that the original source and author(s) are credited.

Link: https://doi.org/10.3897/natureconservation.56.111745.suppl1

Supplementary material 2

Bayesian inference (BI) tree of the 1069-bp cytochrome *b* gene sequences of *Acheilognathus tabira tabira* individuals from the Seto Inland Sea and Ise Bay regions

Authors: Gen Ito, Naoto Koyama, Ryota Noguchi, Ryoichi Tabata, Seigo Kawase, Jyun-ichi Kitamura, Yasunori Koya

Data type: pdf

Explanation note: Numbers at nodes indicate Bayesian posterior probabilities; the value is indicated when it exceeds 0.80.

Copyright notice: This dataset is made available under the Open Database License (http://opendatacommons.org/licenses/odbl/1.0/). The Open Database License (ODbL) is a license agreement intended to allow users to freely share, modify, and use this Dataset while maintaining this same freedom for others, provided that the original source and author(s) are credited.

Link: https://doi.org/10.3897/natureconservation.56.111745.suppl2

Supplementary material 3

The distribution map of Groups estimation using SAMOVA of *Acheilognathus tabira tabira*

Authors: Gen Ito, Naoto Koyama, Ryota Noguchi, Ryoichi Tabata, Seigo Kawase, Jyun-ichi Kitamura, Yasunori Koya

Data type: pdf

Explanation note: Circles show groups (see Fig. 2). Gray areas are the estimated natural distribution area of *A. tabira* 5 subspecies based on Kitamura et al. (2012). This altitude map was used with permission from the Geospatial Information Authority of Japan (https://maps.gsi.go.jp/) and digital national and information of Ministry of Land, Infrastructure, Transport and Tourism (https://nlftp.mlit.go.jp). The paleo-river system follows Kuwashiro (1959).

Copyright notice: This dataset is made available under the Open Database License (http://opendatacommons.org/licenses/odbl/1.0/). The Open Database License (ODbL) is a license agreement intended to allow users to freely share, modify, and use this Dataset while maintaining this same freedom for others, provided that the original source and author(s) are credited.

Link: https://doi.org/10.3897/natureconservation.56.111745.suppl3